

Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

ESTIMATING THE TIME OF DEATH IN RODENT ANIMAL HOUSE FACILITIES

Sara Capas Peneda Vieira Pereira

Orientadora:

Prof. Doutora Margarida Duarte Cerqueira Martins de Araújo

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ABSTRACT

Estimation of the time of death (TOD) is a process that involves the evaluation and correlation of various *post mortem* changes. Surprisingly, there's a lack of information regarding *post mortem* changes in laboratory animals, namely mice (*Mus musculus*) and rats (*Rattus norvegicus*), the species most widely used in biomedical research. Knowing that in rodent animal house facilities it is of the utmost importance to be able to estimate the TOD, the aim of this study was to obtain reference values for the establishment of *rigor mortis*, *algor mortis* and *post mortem* ocular changes in laboratory mice (C57BL/6 and CD1 strains) and rats (Wistar Han) in controlled temperature and relative humidity conditions. All animals were housed and maintained in accordance with Portuguese law (DL nº 113/2013) and international welfare criteria. Cardio-respiratory arrest (CRA) was induced by a single intraperitoneal administration of pentobarbital sodium (300 mg/Kg). *Rigor mortis* was evaluated by flexing and extending the animals' joints (temporomandibular joint, neck, frontlimbs, hindlimbs and tail) and a score from 0 (flexible joint) to 4 (stiff joint) was given according to its rigidity in defined times. Rigidity measurements were performed during the first five and between the 17th and the 27th hours *post mortem*, until the end of the resolution process. Ocular changes were observed throughout the same period of time and given a score from 0 to 4, according to the degree of corneal opacity and sinking of the eyeball. Additionally, *post mortem* decrease in rodents' body temperature was evaluated, allowing us to generate mathematical equations that describe the process of *algor mortis* in mice and rats. Considering that in rodent animal house facilities animals live in controlled temperature and relative humidity conditions, we decided to plot our *rigor mortis* and *post mortem* ocular changes data to a summary timeline chart. These chart may be a useful tool to estimate TOD by animal technicians and researchers, just by evaluating the animals' joint rigidity and ocular changes. We hope that our results contribute to increase the information that we can obtain from each animal, reducing the total number of rodents used in research.

Keywords: Laboratory animals, *rigor mortis*, *algor mortis*, *post mortem*, mouse, rat

This experimental work was conducted at the Rodent Animal House Facility of the Abel Salazar Institute of Biomedical Sciences, Porto University (Biotério ICBAS-UP) and was a part of the final practical training of Sara Capas' integrated master in veterinary medicine studies.

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DEDICATION

À memória da minha mãe, ao meu pai e à Francisca.

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ABBREVIATIONS

ADP – Adenosine diphosphate
ATP – Adenosine triphosphate
ATPase – Adenosine triphosphatase
°C - degree Centigrade (or Celsius)
Ca²⁺ - Calcium ion
CNS – Central nervous system
CRA – Cardiorespiratory arrest
FL – Front limbs
h – Hours
HL – Hind limbs
Hr – Relative Humidity (%)
Kg - Kilogram
mg – milligrams
min – Minutes
Na²⁺ - Sodium ion
RM – *Rigor mortis*
SD – Standard deviation
SEM – Standard error of mean
RT – Room temperature (°C)
PMI – *post mortem* interval
TMJ – Temporomandibular joint
TOD – Time of death

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1. INTRODUCTION

Popular wisdom states that “death is inevitable, although its occurrence is unpredictable”, but when it occurs is important to discover how and when it happened. Estimating the time of death in humans has been a topic of much interest in the field of Forensic Medicine and there are various methods which are based in identifiable alterations that may occur during the *post mortem* interval (PMI), to which a temporal value since death can be attributed (Swift 2006). The literature relating to early PMI estimation is constantly updated with novel or adapted techniques, mainly focusing: *algor mortis*, *rigor mortis*, *livor mortis* and some morphologic (e.g. body staining or swelling, ocular changes, etc.), biochemical, molecular and ultrastructural changes (Swift 2006). Since all of these methods have limitations due to the high variability of factors that influence them, to assess the time of death one should not rely on only one of these processes (Calabuig 2005, Henssge & Madea 2007, Saukko & Knight 2016).

In rodent animal house facilities it is of the utmost importance to estimate the time of death (TOD) in animals that are found dead. It would help to predict the cause of death (together with necropsy findings and histopathologic evaluation) but it could also be very useful to researchers, giving them a precise information regarding their protocol and, if possible, allowing the collection of animal samples, encouraging two of the three R's that laboratory animal sciences promote: Refinement and Reduction. Surprisingly, there is no recent data in the literature indicating the methods that should be used to estimate the time of death in laboratory rodents.

Knowing the importance of determining the time of death of rodents in animal house facilities, we decided to evaluate three different processes that occur in the early PMI: *rigor mortis*, *algor mortis* and ocular changes.

1.1. *Rigor mortis*

Rigor mortis (RM), or *post mortem* rigidity, is usually defined as the stiffening of muscles after death (Calabuig *et al.* 2005, Saukko & Knight 2016).

1.1.1. Muscle contraction and *rigor mortis*

To understand the process of *rigor mortis*, one has to understand the mechanisms of muscle contraction. Skeletal muscle is composed of numerous muscle fibers containing myofibrils – actin and myosin – that are arranged in sarcomeres, giving the muscle its striated appearance. Each sarcomere is composed of A bands, I bands and Z disks. Each Z disk separates each sarcomere from the other (Figure 1) (Reece 2015, Hall 2016).

Muscle contraction occurs when an action potential arrives at the muscle fiber membrane, depolarizing T-tubules (invaginations of the muscle fiber membrane to the interior of the muscle fiber), causing the sarcoplasmic reticulum to release previously stored calcium ions to the sarcoplasm, the muscle fiber cell cytoplasm. These calcium ions will surround the myofibrils and

initiate the attractive forces between the actin and myosin filaments (Mescher 2013, Reece 2015, Hall 2016).

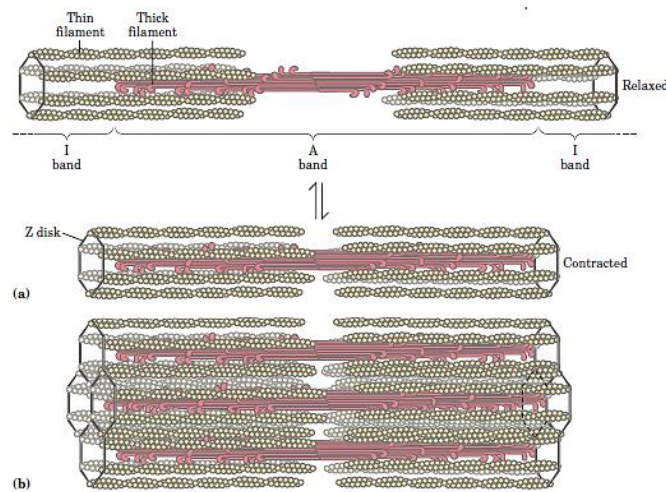


Figure 1 – Contraction of a sarcomere. It is possible to identify the different areas of a sarcomere (I band, A band and Z disk) and to observe the sliding of filaments during the process of contraction (Nelson & Cox 2012).

Actin filaments are composed of actin, tropomyosin and troponin. The first one is arranged as a helix and the second is organized spirally around the actin helix. The third one is composed of three subunits (I, T, C) which are attached intermittently along the sides of the tropomyosin molecules. These subunits have different affinities: 1) troponin I has a strong affinity for actin; 2) troponin C for calcium ions and 3) troponin T for tropomyosin. Myosin filaments are composed of multiple myosin molecules, constituted by two heavy chains and four light chains that form the head, body and arms of the myosin filament. The arms extend the head outward the body forming the cross-bridges which are going to interact with actin (Figure 2) (Mescher 2013, Reece 2015, Hall 2016).

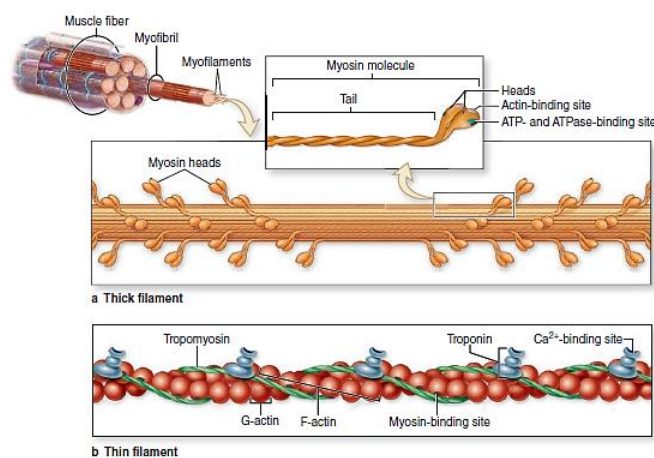


Figure 2 – Composition of myofibrils. It is possible to observe a) the composition of myosin filaments (thick filaments) and b) the composition of actin (thin filament) (Mescher 2013).

The myosin heads have ATPase activity which allows the cleavage of ATP molecules to produce energy for the process of contraction. The tropomyosin-troponin complex inhibits the binding between actin and myosin which is necessary for the process of muscle contraction. As mentioned earlier, troponin C has a high affinity to calcium ions and combines with them to “activate” the actin filament. According to this mechanism, when calcium ions bind to troponin C, the troponin complex undergoes a conformational change that will uncover the active sites of actin, thus allowing the interaction between actin and myosin molecules (Reece 2015, Hall 2016, Hall 2016).

ATP is the source of energy involved in muscle contraction. Before contraction begins, the heads of the cross-bridges bind with ATP which, due to the ATPase activity of the myosin heads, is cleaved in ADP and phosphate that remains attached to the head. At this phase, there’s no interaction between actin and myosin due to the blockage of actin active sites, but this is surpassed when troponin-tropomyosin complex binds calcium ions. As the myosin heads attach to the active sites of actin profound changes in the intramolecular forces between the head and arm occurs; the new alignment of force causes the head to tilt toward the arm and to drag the actin filament along with it. The energy for this process is originated by the earlier cleavage of ATP by the myosin head. Once this happens, the ADP molecule is released and a new molecule of ATP binds to the myosin head allowing the detachment of the head from the actin. This process is repeated until the end of the process of muscle contraction, being this theory known as “Walk-along theory of contraction” (Reece 2015, Hall 2016).

Relaxation is achieved by the removal of calcium ions from the sarcoplasm. Calcium channels in the sarcoplasmic reticulum wall will pump calcium ions from the sarcoplasm to the interior of the sarcoplasmic reticulum by active transport with consumption of ATP. The amount of ATP present in the muscle fiber is only sufficient to maintain full contraction for only 1 to 2 seconds. In skeletal muscle, there are three sources of ATP: 1) the substance phosphocreatine which is used to reconstitute the ATP; 2) glycogen reserves in the muscle which are used to reconstitute ATP and phosphocreatine and 3) the process of oxidative phosphorylation. It is important to note that the second one can occur in the absence of oxygen, through the anaerobic glycolysis with the formation of lactic acid (Reece 2015, Hall 2016, Hall 2016).

After death there’s some production of ATP by anaerobic glycolysis, but ultimately the storage of ATP is depleted. As mentioned earlier, ATP is necessary to the detachment of the myosin heads from the active sites of actin, consequently, when there’s no ATP available, it’s not possible to detach the cross-bridges from actin and thus contraction is maintained originating muscular rigidity - the main feature of *rigor mortis* (Hall 2016, Krompecher 2016). *Rigor mortis*

affects all muscles including diaphragm, myocardium, smooth muscle of the bladder, seminal vesicles and pupils, that may maintain different sizes after death (Saukko & Knight 2016).

1.1.2. *Rigor mortis* phases

It is possible to divide the whole process of *rigor mortis* in four different phases: 1) delay period; 2) onset period; 3) *rigor* and 4) resolution.

1) Delay period: the muscle still has the possibility to produce ATP, so there's sufficient energy to split the actin-myosin cross-bridges. ATP depletion depends on its muscle content at the time of death, on the possibility of *post mortem* ATP production and the rate of ATP hydrolysis. It correlates with the increase of muscle stiffness (Krompecher 2016).

2) Onset period: ATP levels fall below a critical threshold, actin-myosin cross-bridges remain intact and rigidity appears. This is a reversible state and if there's addition of ATP or oxygen, the muscle may still relax (Krompecher 2016) .

3) *Rigor* phase: rigidity increases and reaches its maximum. This state is irreversible due to *post mortem* modifications of muscle fibres that destroy the muscle ability to relax (Krompecher 2016).

4) Resolution phase: The process of resolution is not completely understood but it is known to occur by processes of autolysis (Krompecher 2016). It is described that enzymes such as μ -calpains are responsible for the degradation of various muscles proteins such as titin, nebulin, desmin and troponin-t (Lonergan *et al.* 2010). Furthermore, cathepsins (acid proteases located in lysosomes) also have a role on proteolysis of muscle after *rigor* (Chéreta *et al.* 2007).

In human cadavers, *rigor mortis* is described to begin as soon as 2-4 hours *post mortem*, reaching maximum intensity at 6-12 hours *post mortem*. Full *rigor* can be maintained during 18-36 hours *post mortem* (DiMaio & DiMaio 2001, Saukko & Knight 2016). Although there are no standardized values for the establishment of RM in laboratory rodents, there are some published data regarding the evolution and resolution of *rigor mortis* in rats. Krompecher (1981) published the following data regarding the process of *rigor mortis* in hind limbs of rats at various temperatures.

- At 37 °C:
 - *Rigor* was fully developed 3 hours *post mortem* and the resolution was completed 6 hours after
- At 24°C:
 - *Rigor* was fully developed 5 hours *post mortem* and resolution was completed 16 hours after

- At 6 °C:
 - *Rigor* was fully developed at 48-60 hours *post mortem* and resolution was only completed 168 hours after (Krompecher 1981).

Krompecher (2016) also studied whether the Nysten's law (that states that *rigor mortis* has a craniocaudal progression) applied to rats and obtained the following values regarding the evolution of *rigor mortis* in that specie:

- *Rigor* was fully developed at:
 - 2 hours *post mortem* in the masticatory muscles
 - 3 hours *post mortem* in the neck
 - 5 hours *post mortem* in front limbs
 - 5 hours *post mortem* in the hind limbs (Krompecher 2016).

Unfortunately, there's no information regarding room temperature (RT) measurements when these data were obtained.

1.1.3. *Rigor mortis* muscular sequence

The sequence of *rigor mortis* muscular involvement was first described by Nysten in 1811. The process was described as being a sequential event affecting initially the muscles of the eyelids, jaw and neck, progressing downwards to affect limb muscles. The cause of such sequence was hypothesised by Shapiro in 1950; he suggested that the process of *rigor* starts at the same time in all muscles, but since there are volume differences the first muscles to be affected are the smallest, where the process can occur faster (Mathur & Agrawal 2011, Vij 2011). Recent studies have disproven this theory (Kobayashi *et al.* 1996, Kobayashi *et al.* 2001) and it is now accepted that *rigor* develops more rapidly in red muscles when compared to white muscle. All muscles are composed of various types of muscles fibres, of which three different types can be identified: 1) type I; 2) type IIa and 3) type IIb (Mescher 2013). The major characteristics of the different muscle fibres are summarized in Table 1.

According to Kobayashi, sequential development of *rigor mortis* in the different muscle groups is related to their glycogen content, that differs between types of muscle fibres. Nevertheless, this theory remains to be proven (Kobayashi *et al.* 1999, Kobayashi *et al.* 2004, Saukko & Knight 2016).

Table 1 – Major characteristics of muscle fibre types (Mescher 2013).

Major characteristics muscle fibre types			
	Type I	Type IIa	Type IIb
Mitochondria	Numerous	Numerous	Sparse
Capillaries	Numerous	Numerous	Sparse
Fibre diameter	Small	Intermediate	Large
Size of motor unit	Small	Intermediate	Large
Myoglobin content	High	High	Low
Glycogen content	Low	Intermediate	High
Major source of ATP	Oxidative phosphorylation	Oxidative phosphorylation	Anaerobic glycolysis
Glycolytic enzyme activity	Low	Intermediate	High
Rate of fatigue	Slow	Intermediate	Fast
Myosin-ATPase activity	Low	High	High
Speed of contraction	Slow	Fast	Fast
Typical major locations	Postural muscles of back	Major muscles of limbs	Extraocular muscles

1.1.4. Factors affecting the time course and intensity of *rigor mortis*

Several factors may influence the time course of RM, but one of the most documented is temperature. It's generally established that lower temperatures delay the process whereas higher temperatures accelerate it, with the exception of extremely low temperatures that can originate *cold stiffening* and extremely high temperatures which originate *heat stiffening* (Krompecher 1981, Calabuig *et al.* 2005, Ozawa *et al.* 2013, Saukko & Knight 2016). The first occurs when the ambient temperature is below -5°C, because with this conditions body fluids freeze and subcutaneous tissues solidify, originating a state of false rigidity. The latter occurs in extremely high temperatures and originates denaturation and coagulation of muscle proteins, causing muscular stiffness (Saukko & Knight 2016).

Another factor that has to be taken into consideration is the physical exercise that occurred before death, since glycogen content of the muscles has a direct influence in RM onset. So it is logical to assume that when death occurs following a period of physical exercise, the glycogen contents will be depleted, with a decrease of the muscle pH (Calabuig *et al.* 2005, Saukko & Knight 2016). Krompecher studied the influence of physical exercise before death in rat muscles and concluded that it shortens the delay period, the onset of *rigor* is faster and its intensity is higher. No differences were observed in the resolution process (Krompecher & Fryc 1978). Furthermore, RM mortis can be broken by forceful movements of extension and flexion of the joints. The same author studied the influence of breaking *rigor* in rat muscles at different PMI and concluded that *rigor* can reappear if broken during the onset period, before rigidity is completely established. The process of breaking RM does not affect its time course, but rigidity is not as

intense when compared to the control group. Once more, the process of resolution was not affected (Krompecher *et al.* 2008). The reappearance of RM after its disruption can be explained by the fact that muscles are composed of numerous muscle fibres of different types, as mentioned earlier, so different fibres may be in different stages of rigidity. Thus, the rigidity that reappears is weaker due to the number of muscles fibres that were broken during the process (Krompecher 2016).

Other factors that could affect RM time course, intensity or both are:

- Age: In old and very young animals the process is usually faster and less intense;
- Muscle mass and nutritional status: The process is usually slower and more intense in individuals with higher muscle mass and faster and weaker in individuals with low muscle mass and cachexia;
- Cause of death, in cases of:
 - . sudden and violent deaths, rigidity appears later and is more intense
 - . prolonged disease, usually the process of *rigor* is faster and weaker
 - . electrocution, the rigidity is faster and more intense
 - . brain or medulla lesions, the process starts earlier
 - . convulsions prior to death, rigidity appears earlier, its more intense and lasts longer.
 - . pathologies that raise body temperature (infectious processes, hyperthermia, loss of body temperature control due to brain lesions), RM is accelerated (Krompecher & Bergerioux 1988, Calabuig *et al.* 2005, Tsokos 2005, Saukko & Knight 2016).

Some authors refer the existence of cadaveric spasm or instantaneous *rigor* (Pirsch *et al.* 2013, Saukko & Knight 2016), but there's a great debate whether it really exists or not since there's no physiological or biochemical explanation for such happening (Tsokos 2005, Bedford & Tsokos 2013). Essentially, this is a form of instantaneous *rigor* that develops without delay period. This is described to occur in deaths following intense physical or emotional stress and seems to be initiated by motor nerve action. It usually affects only one group of muscles but could become a generalized process (Calabuig *et al.* 2005, Saukko & Knight 2016). Calabuig *et al.* (2005) also describes some causes of death that could be related to this phenomenon, such as convulsions prior to death, wounds, spontaneous lesions of the CNS, mechanical asphyxiation and drowning (Calabuig *et al.* 2005).

1.2. *Algor mortis*

Rodents are homoeothermic animals that have a constant body temperature, maintained due to an equilibrium between heat production and heat loss. Heat production occurs as a consequence of basal rate cellular metabolism, muscular activity and hormonal activity (e.g.

mediated by thyroxine). It is mainly produced by internal organs (brain, liver, heart and skeletal muscles) and then distributed to the skin and throughout the body by blood circulation (Smart & Kaliszan 2012, Hall 2016). Blood flow is very important to control body temperature, since vasodilation promotes heat loss whereas vasoconstriction promotes heat maintenance. The skin, subcutaneous tissues and fat act together as heat insulators of the body, due to their poor heat conductivity properties (Smart & Kaliszan 2012, Hall 2016). These processes cease to exist after death and the body will have a tendency to lower its temperature to equal ambient temperature. A body will lose heat to its surroundings in four forms: 1) by conduction (objects in contact with the body that absorb heat); 2) radiation (in the form of infrared rays); 3) convection (air movement that dissipates heat) and 4) evaporation (liquid vaporization) (Hall 2016). A dead body will mainly lose heat by conduction and convection (Tsokos 2005).

1.2.1. Factors affecting the cooling curve

The cooling curve of a dead body occurs because there is the formation of a temperature gradient between the dead body and the environment, so body temperature decrease is proportional to ambient temperature gradient. But besides ambient temperature, air movement and humidity can also influence the cooling curve. Since the body loses heat by convection and conduction with the adjacent air, air movement will maintain a temperature gradient while still air will maintain a layer of warm air around the body, diminishing the temperature gradient (Tsokos 2005).

Since body dimensions are related to body surface and a dead body will lose heat mainly by conduction and convection, it is logical that body dimensions will affect the temperature gradient and consequently, the cooling curve. Along these, the presence of variable amounts of fat will also influence the process of losing heat, since fat has insulating properties. So, higher body dimensions are related to slower cooling. Some authors also point that since generally humans of the female gender have a higher proportion of body fat, cooling could be slower in cases of female bodies (Tsokos 2005, Saukko & Knight 2016). The posture of a dead body will also influence the cooling curve, since it is related to the amount of skin that is in contact with a determined surface and/or air. Thus, a curled up body will lose heat slower than a body lying on its back (Tsokos 2005, Saukko & Knight 2016). And, finally, the initial body temperature which cannot be assumed to be the same in every animal. In normal conditions body temperature varies along the day, but in pathological conditions cases of hyperthermia and hypothermia can precede death, influencing the cooling curve (Saukko & Knight 2016).

1.2.2. *Algor mortis* as a tool to estimate the time of death

In the last century, there have been numerous approaches to the use of cooling rate of a dead body as a tool to estimate the time of death (Kaliszan *et al.* 2009). The first approaches

were made by Rainy in 1868; he was the first to apply Newton's law of cooling to a dead body and to consider the influence of the ambient temperature. He did serial measurements of rectal temperature and established the gradient of the curve of temperature decrease *versus* time. This author also noted the existence of an interval of time immediately after death during which the rate of decrease in body temperature was slower, but the term plateau was only introduced in 1965 by Shapiro. Since then, there have been numerous studies about the use of different body sites to measure temperature (rectum, liver, brain, muscle, skin) and various mathematical approaches to the cooling curve as single exponential, second exponential and triple exponential mathematical equations (Kaliszan *et al.* 2009). Thermodynamically, the human body has been compared to an infinite cylinder, with heat being lost radially and not axially, establishing a temperature gradient from the interior to the surface. Thus, in the beginning, the gradient established between the core of the body and its surface is small and core body temperature is maintained stable. But the gradient tends to get higher as skin temperature gets lower, making the core body temperature decrease at a faster rate. In the end the gradient is small, so the rate of cooling declines. Consequently, the cooling curve has a sigmoid shape which contrasts with the exponential cooling curve of a body who obeys the Newton's law of cooling. The presence of a plateau (arguable, since some authors didn't observe this phenomenon) could be attributed to: 1) some residual metabolic activity after death, including the process of *rigor mortis* because the muscular contraction will produce heat; 2) physical properties of the body and 3) the thermodynamic characteristics of the body (Al-Alousi 2002, Smart & Kaliszan 2012).

Knowing that body temperature measurements change according to the site where they are taken, non-conventional sites like the outer ear, the eyeball and soft tissues of the orbit were recently tested. These sites seem to have the advantage of not presenting a temperature plateau, so the cooling rate could be expressed as a single exponential equation as in the Newton's Law of cooling (Kaliszan *et al.* 2005, Kaliszan *et al.* 2009, Kaliszan *et al.* 2010). One of the most widely used methods is the nomogram developed by Henssge (based in rectal temperature measurements), which takes into account the body mass and other corrective factors such as the presence of clothing (Henssge & Madea 2004). Nevertheless, the numerous factors and conditions to which a cadaver may be subject *post mortem* remain as the main limitation of this approach (Calabuig *et al.* 2005, Saukko & Knight 2016).

There are some published data regarding the process of *post mortem* cooling in rats, using internal probes. Jones (1982) obtained continuing body temperature measurements in rats with body weights ranging from 230 to 270 g by the application of a subcutaneous probe. The probes were implanted before the moment of death and measurements were made until body temperature equalled room temperature, approximately 6 hours *post mortem* (Jones 1982). Henriquez *et al.* (2013) obtained continuing measurements of body temperature in rats with a

mean body weight of 400 g using a transesophageal probe. They concluded that body temperature of rats equalled the room temperature (~ 20°C) at 11 hours and 14 minutes *post mortem* (Henriquez *et al.* 2013).

1.3. *Post mortem* ocular changes

The main macroscopic ocular changes that have been described after death are corneal opacity and flaccidity of the eyeball. *In vivo*, corneal transparency is dependent of its physical structure and on mechanisms that maintain its state of relative dehydration. Mouse and rat cornea is composed of five layers - epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium – similar to the human cornea (Hofstetter *et al.* 2006, Treuting *et al.* 2012). To maintain its optimal transparency cornea is avascular, so it depends on aqueous humor, limbal capillaries and tears to supply its energy needs (Gum & Mackay 2013). Besides providing nutrients to the avascular cornea, the lacrimal film also maintains an optical uniform surface and lubricates the cornea, helps to remove foreign materials from the cornea and conjunctiva and controls the local bacterial flora (Gum & Mackay 2013). At the time of death tear production ends and if the eyelids remain open ocular surface damage is facilitated by ambient factors, ultimately leading to opacification of the cornea. In human cadavers corneal clouding could be visible 45 minutes *post mortem*, but usually it is only evident 4 hours after death, if the eyelids remain open. But if the eyelids remain closed corneal clouding can be observed as late as 24 h *post mortem*, indicating that corneal alterations time course is variable, depending on eyelids position (Calabuig *et al.* 2005). Corneal opacity can be used as a tool to estimate the time of death, but its evaluation is subjective, even if an image analysis method or an objective score is used (Zhou *et al.* 2010, Kawashima *et al.* 2015).

Intraocular pressure is directly dependent of arterial blood pressure, so it starts to decline after death, leading to flaccidity of the eyeball (Gum & Mackay 2013, Saukko & Knight 2016). This flaccidity is also due to evaporation of intraocular fluid and consequently its chronological sequence depends on the position of the eyelids (Calabuig *et al.* 2005). There are some reports indicating that intraocular pressure can be used to estimate the time of death using a tonometer, but this approach is not very frequent (Balci *et al.* 2010).

2. OBJECTIVES

Knowing that there is a lack of information in the literature regarding *post mortem* processes in laboratory rodents, the aim of this study was to obtain standardized values of several parameters related to *post mortem* processes (*rigor mortis*, *algor mortis* and *post mortem* ocular changes) that would allow the estimation of TOD in laboratory mice (*Mus musculus*) and rats (*Rattus norvegicus*).

3. MATERIALS AND METHODS

3.1. Animals

In this study we used rats and mice that were involved in previous protocols but had a full recovery or maintained a normal health condition, according to veterinary evaluation.¹¹ *Rattus norvegicus* (Wistar Han rats, weighting 235 – 535 g with 2 to 8 months old of both genders) and 26 *Mus musculus* – 11 CD1 mice (weighting 27 – 55 g body weight with 2 to 11 months old of both genders) and 15 C57BL/6 mice (weighting 23 – 34 g body weight with 2 to 9 nine months old of both genders) were maintained at the Rodent Animal House Facility of ICBAS-UP. All animals were kept in IVC cages with proper ventilation, stable temperature (20-24 °C) and relative humidity (40-60%) intervals, in accordance with the specie requirements. A regular 12 h light /dark cycle and *ad libitum* access to food (standard diet) and fresh water were also assured. All animal procedures were performed according to the Portuguese DL nº 113/2013 and European Guidelines for humane and responsible animal care.

3.2. Rigor mortis Evaluation

Cardio-respiratory arrest (CRA) was induced by a single intraperitoneal administration of pentobarbital sodium (400 mg/kg). After CRA, the animals were put in a flat surface (an acrylic table) in dorsal recumbence. All animals were kept at constant room temperature and humidity conditions during this experiment. *Rigor* was evaluated manually by flexing and extending the temporomandibular joint (TMJ), neck, front limbs (FL), hind limbs (HL) and tail. A score from 0 to 4 (Figure 3) was attributed to each joints in pre-established time points. Measurements were made each five minutes in the first hour, each 10 minutes in the 2nd hour and each 15 minutes in the following 3rd and 4th hours. Photographs were taken at 0; 0.5; 1; 2; 3; 4; 18 and 24 hours after CRA. For this purpose, the body of the animal was suspended by the thoracoabdominal region with the aid of elastic bands.

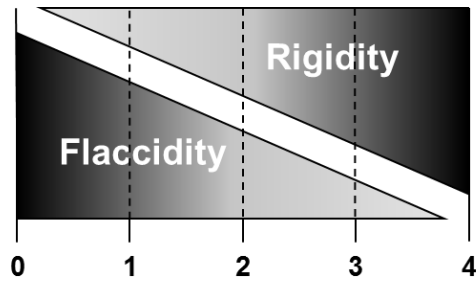


Figure 3 – Classification system (score from 0 to 4) for *post mortem* rigidity.

3.3. *Algor mortis* evaluation

Microchip programmable temperature transponders (BMDS IPTT-100, Plexx BV, 6660 AE ELST, Netherlands) were implanted subcutaneously in the right flank at the moment of CRA (t=0). Temperature readings were taken with the Pocket Scanner System DAS 5007 (Bio Medic Data System [BMDS], Seaford, DE) every 5 minutes in the first hour *post mortem*, every 10 minutes in the second hour and then every 15 minutes until the end of the experiment. Data was analyzed with GraphPad Prism™ (version 6.00 for Windows, GraphPad Software, La Jolla California USA).

3.4. *Post mortem* ocular changes evaluation

The animals' eyelids remained open and were evaluated 0; 15; 30; 60; 120; 180; 240; 1020; 1140 and 1260 minutes after CRA. The eyeballs were observed and given a score from 0 to 4 (Table 2), according to the degree of eye opacity and sinking.

Table 2 – Classification system for *post mortem* eyeball changes.

Score	Eyeball Changes
0	Normal
1	Mild corneal opacity
2	Total corneal opacity
3	Mild sinking
4	Total sinking

3.5. Statistical Analysis

All the data are reported as the sample mean \pm the standard error of mean (SEM). Graphics were created using GraphPad Prism™ (version 6.00 for Windows, GraphPad Software, La Jolla California USA).

4. RESULTS

4.1. Post mortem evaluation of C57BL/6 mice

All experiments were conducted with controlled room temperature (RT) and relative humidity (RH) conditions: RT = $21 \pm 0,1^{\circ}\text{C}$ and RH = $27,2 \pm 0,3 \%$; $n=257$.

4.1.1. Rigor mortis evaluation

The results show that TMJ was completely blocked in the first hour after CRA ($0,9 \pm 0,1$ h), followed by the neck ($1,3 \pm 0,1$ h) and front limb ($1,9 \pm 0,2$ h) joints. The tail ($2,2 \pm 0,2$ h) and hind limb ($2,7 \pm 0,4$ h) joints were completely rigid three hours *post mortem* (Figure 4A). After full RM was achieved, muscle stiffness was maintained until approximately twenty hours *post mortem*.

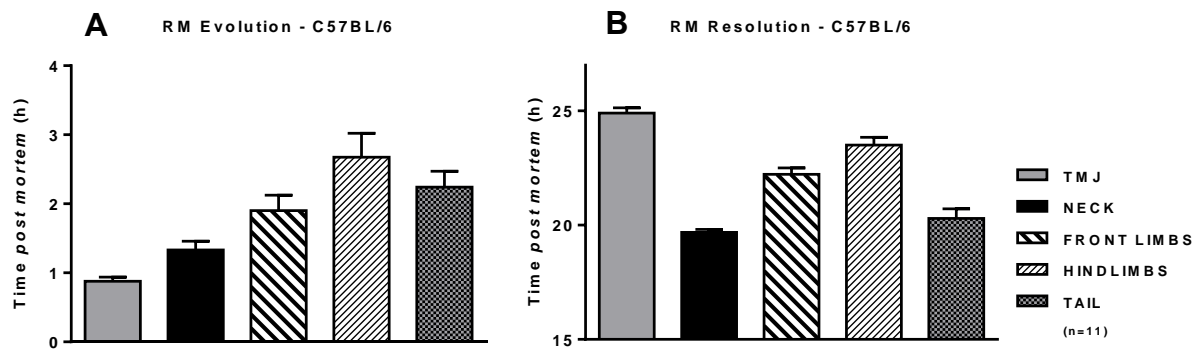


Figure 4 – Rigor mortis full evolution and resolution chronology (hours, Mean + SEM) in C57BL/6 mice ($n=11$).

The resolution process began approximately 17 hours *post mortem* (Figure 5). This process was completed first at the neck ($20 \pm 0,1$ h) followed by the tail ($20 \pm 0,4$ h), front limbs ($22 \pm 0,3$ h), hind limbs ($24 \pm 0,4$ h) and finally the TMJ ($25 \pm 0,2$ h) that completed RM resolution process (Figure 4B).

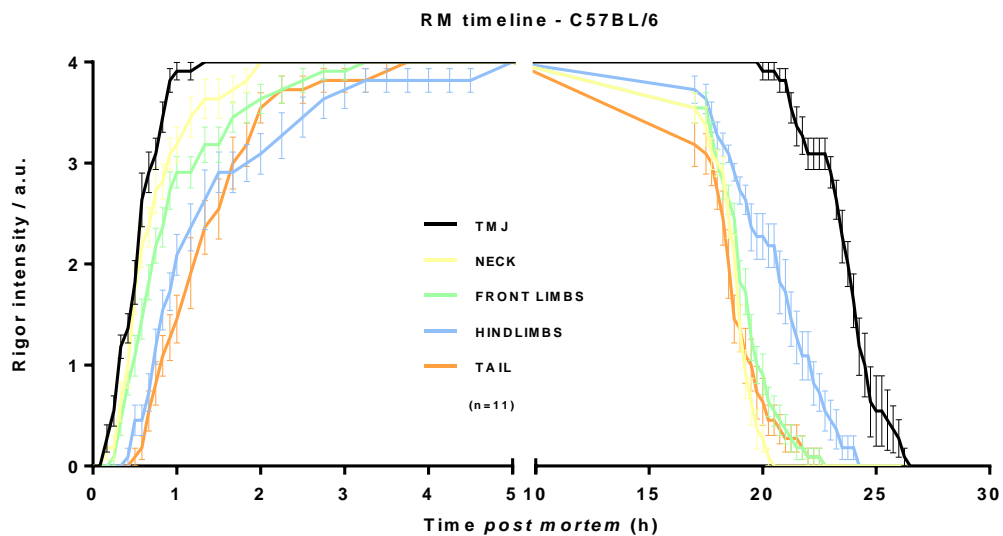


Figure 5 – Rigor mortis complete timeline (Mean \pm SEM) in C57BL/6 mice ($n=11$).

4.1.2. *Post mortem* body posture evaluation

The progressive nature of *rigor mortis* regarding body posture was recorded photographically over time (Figure 6). The maximum rigidity was observed between 3 and 4 hours *post mortem*, in agreement with previously recorded RM data (Figure 4 and 5).



Figure 6 – *Post mortem* body posture timeline in C57BL/6 mice ($n=11$).

4.1.3. *Post mortem* ocular changes evaluation

In C57BL/6 mice ocular changes begins with a whitish cornea 15 minutes after CRA (17 ± 2 min, Figure 7B) and evolves to its complete opacity (Figure 7C) during the first *post mortem* hour (49 ± 5 min). Although the eye remains “white”, mild sinking of the eyeball (observed during the second *post mortem* hour, 83 ± 11 min, Figure 7D) attenuates the colour contrast in this dark fur animals. Evaluation of cornea colour becomes difficult after total sinking of the eyeball, a process that occurs about 22nd hours *post mortem* (1140 ± 39 min, Figure 7E).

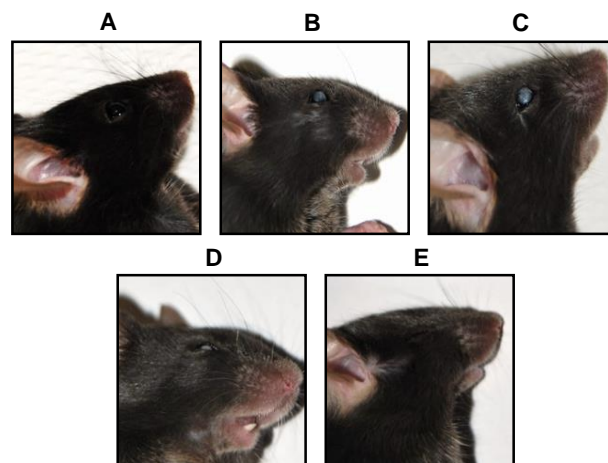


Figure 7 – *Post mortem* ocular changes in C57BL/6 mice. A - Normal eye; B - Mild opacity; C - Total opacity; D - Mild sinking of the eyeball and E - Total sinking of the eyeball ($n=11$).

4.1.4. *Algor mortis* evaluation

Body temperature measurements were assessed in controlled room temperature and relative humidity conditions ($RT = 21 \pm 0,1^{\circ}\text{C}$ and $RH = 27,2 \pm 0,3 \%$; $n=257$). The mice's body cooling rate was higher in the first hour after CRA, but their body temperature equalled room temperature 3 hours *post mortem* ($3 \pm 0,4$ h) (Figure 8). The initial body temperature was $34,3 \pm$

0,3 °C but it decreased abruptly to $25,1 \pm 0,3$ °C in the beginning of the first hour, reaching $22,8 \pm 0,3$ three hours *post mortem*. This results indicate that body temperature decrease rate exhibits an exponential decay. By nonlinear regression analysis we were able to obtain the mathematical equation of the exponential cooling curve, shown in Figure 8.

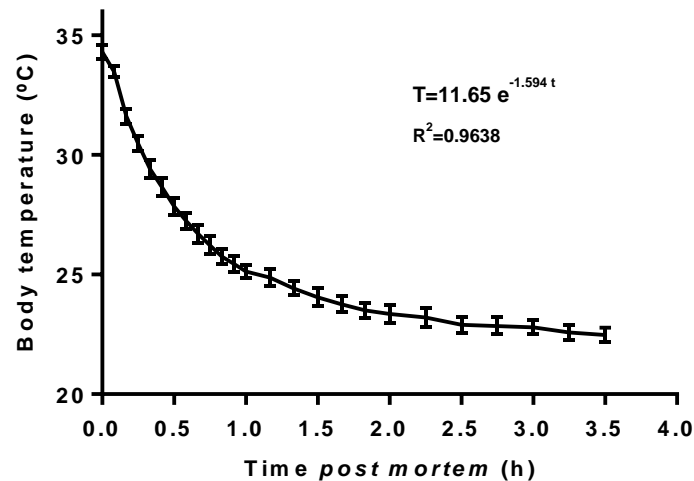


Figure 8 – *Post mortem* body temperature timeline in C57BL/6 mice (mean \pm SEM, $n=4$).
T=body temperature (°C) and t=time(hours).

4.2. *Post mortem* evaluation of CD1 mice

All experiments were conducted with controlled RT ($21 \pm 0,1$ °C, $n=141$) and RH ($30 \pm 0,4$ %, $n=141$) conditions.

4.2.1. *Rigor mortis* evaluation

TMJ was completely blocked in the second hour *post mortem* ($1,6 \pm 0,1$ h), followed by the neck ($2,1 \pm 0,2$ h) and front limbs ($2,4 \pm 0,2$ h) during the next hour. The tail ($2,9 \pm 0,1$ h) and hind limb ($3,3 \pm 0,2$ h) joints were completely rigid fourth hours after CRA (Figure 9A). After full RM was achieved, muscle stiffness was maintained until approximately twenty-one hours *post mortem* (Figure 10).

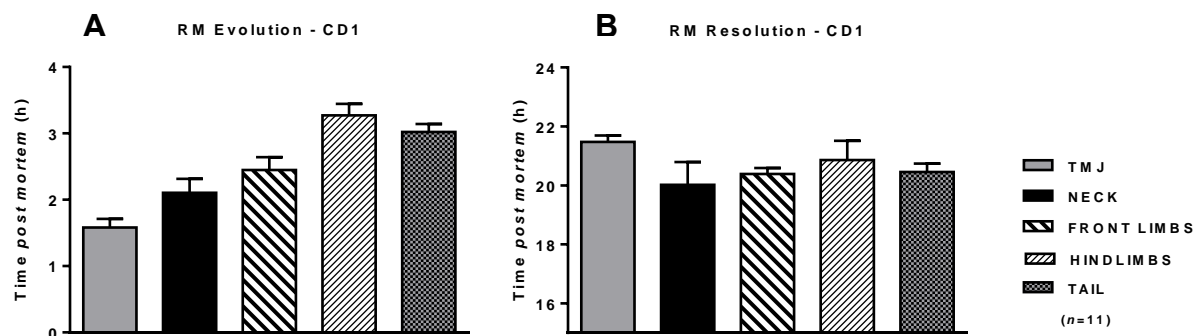


Figure 9- *Rigor mortis* full evolution and resolution chronology (hours, Mean + SEM) in CD1 mice ($n=11$).

The resolution process was completed in the neck ($20,0 \pm 0,2$ h) followed by the front limbs ($20,4 \pm 0,2$ h), tail ($20,5 \pm 0,3$ h), hind limbs ($20,9 \pm 0,2$ h) and finally the TMJ ($21,5 \pm 0,2$ h). RM resolution was completed 22 hours *post mortem* (Figure 9B).

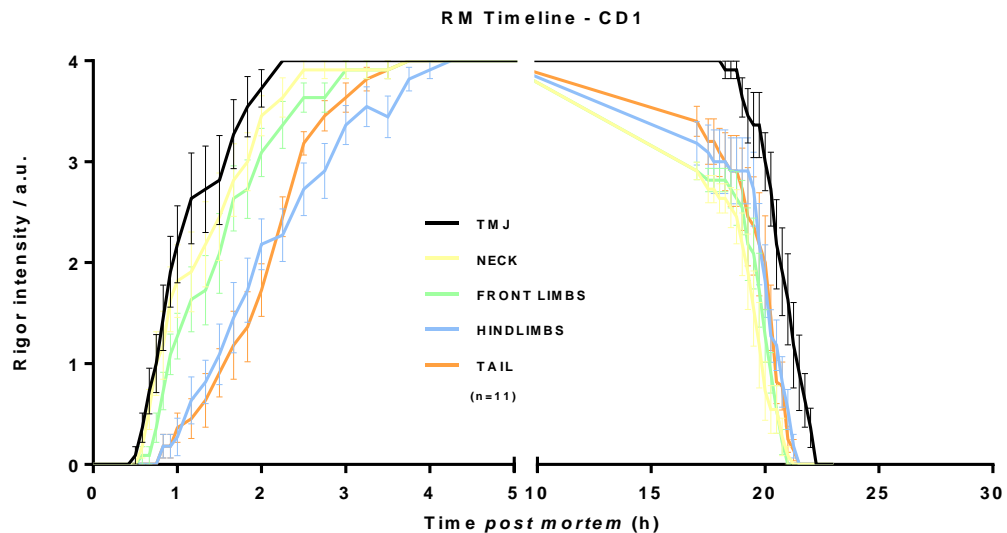


Figure 10 - Timeline of the complete process of *rigor mortis* (Mean \pm SEM) in CD1 mice ($n=11$).

4.2.2. *Post mortem* body posture evaluation

Post mortem CD1 body posture was recorded photographically over time (Figure 11). The maximum rigidity was observed between 3 and 4 hours *post mortem*, in agreement with previously recorded RM data (Figure 9 and 10).

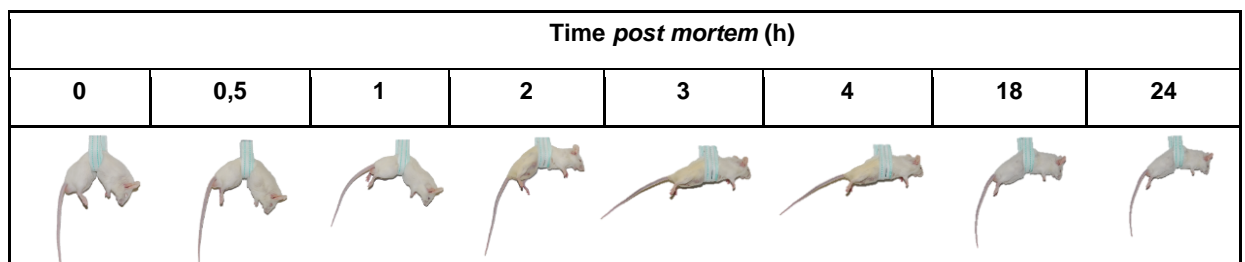


Figure 11 - *Post mortem* body posture timeline in CD1 mice ($n=11$).

4.2.3. *Post mortem* ocular changes evaluation

In CD1 mice ocular changes begins with a whitish cornea 30 minutes after CRA (26 ± 2 min, Figure 12B) and evolves to its complete opacity (Figure 12C) during the first hour *post mortem* (60 ± 6 min). Mild sinking of the eyeball (observed during the second hour *post mortem*, 120 ± 10 min, Figure 12D) evolves to total sinking 19 hours *post mortem* (1149 ± 9 min, Figure 12E).

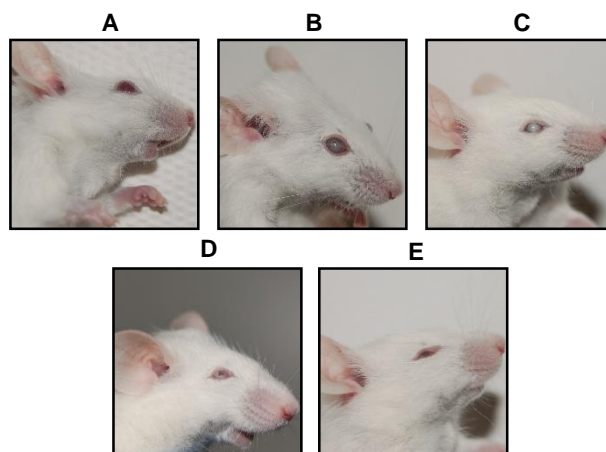


Figure 12 – *Post mortem* ocular changes in CD1 mice. A – Normal eye; B- Mild opacity; C – Total opacity; D – Mild sinking of the eyeball E- Total sinking of the eyeball ($n=11$).

4.2.4. *Algor mortis* evaluation

Body temperature measurements were assessed in controlled room temperature and relative humidity conditions ($RT = 21 \pm 0,1$ °C and $RH = 30 \pm 0,4$ %; $n=141$). Similar to C57BL/6 mice, CD1 mice's body cooling rate was higher in the first hour after CRA, but their body temperature equalled room temperature 3 hours *post mortem* ($3,1 \pm 0,2$ h, Figure 13). The initial body temperature was $34,6 \pm 0,3$ °C but it decreased to $24,9 \pm 0,2$ °C in the beginning of the first hour *post mortem*, reaching $21,5 \pm 0,3$ °C at the beginning of the third hour *post mortem*. This results indicate that body temperature decrease rate exhibits an exponential decay compatible with Newton's law of cooling. By nonlinear regression analysis, we were able to obtain the mathematical equation of the exponential cooling curve, shown in Figure 13.

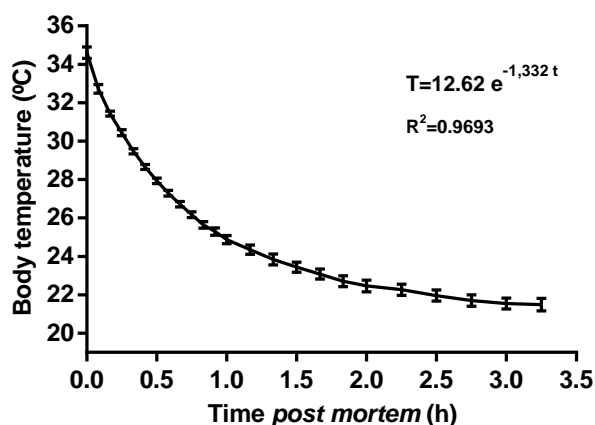


Figure 13 - *Post mortem* body temperature timeline in CD1 mice (*mean* \pm *SEM*, $n=10$).
T=body temperature (°C) and t=time(hours).

4.3. *Post mortem* evaluation of Wistar Han rats

All experiments were conducted with controlled RT ($21 \pm 0,1$ °C, $n=186$) and RH ($29 \pm 0,3$ %, $n=186$) conditions.

4.3.1. Rigor mortis evaluation

The results show that TMJ was completely blocked in the second hour after CRA ($1,7 \pm 0,1$ h), followed by the neck ($2,2 \pm 0,2$ h) and front limb ($2,4 \pm 0,2$ h) joints. The tail ($2,7 \pm 0,2$ h) and hind limb ($3,1 \pm 0,2$ h) joints were completely rigid before three hours post mortem (Figure 14A). After full RM was achieved, muscle stiffness was maintained until approximately twenty-one hours post mortem (Figure 15).

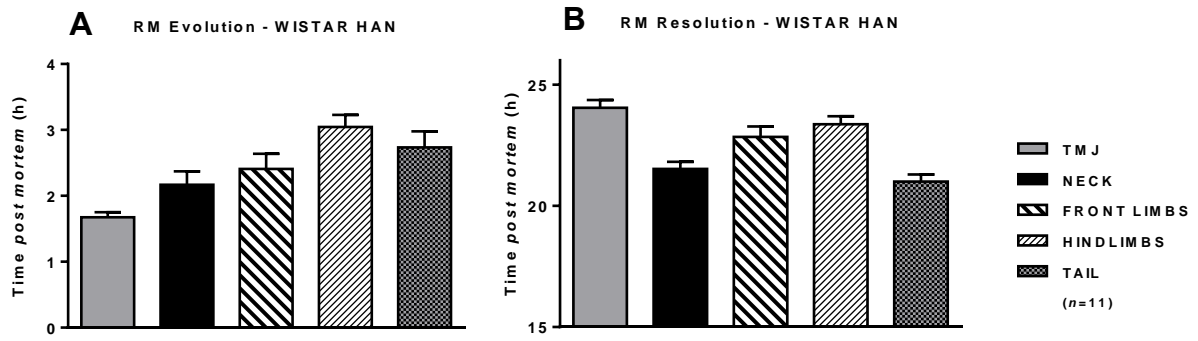


Figure 14 - Rigor mortis full evolution and resolution chronology (hours, Mean + SEM) in Wistar Han rats ($n=11$).

The resolution process began approximately 17 hours *post mortem* (Figure 15). This process was completed first at the tail ($21 \pm 0,3$ h) followed by the neck ($22 \pm 0,3$ h), front limbs ($23 \pm 0,4$ h) and hind limbs ($23 \pm 0,3$ h), and finally the TMJ at ($24 \pm 0,3$ h) that completed RM resolution process. (Figure 14B).

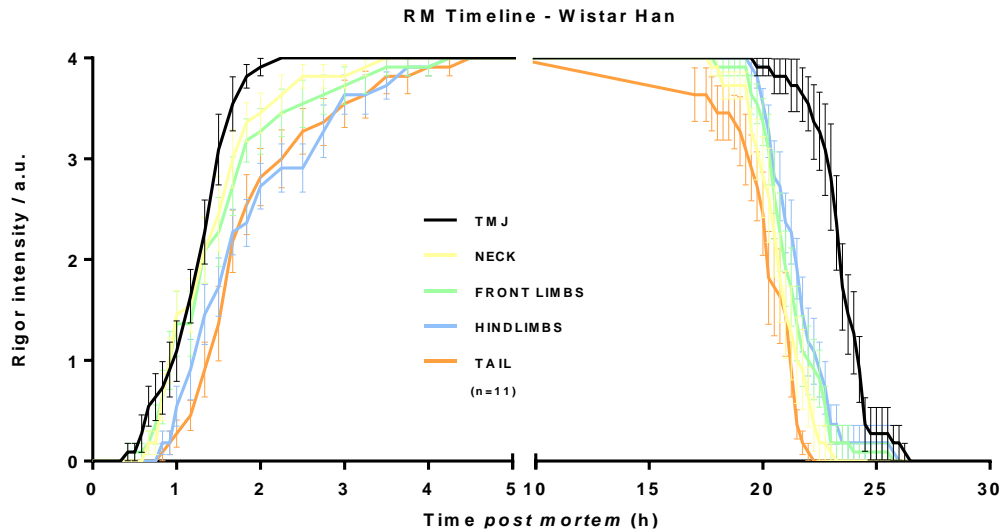


Figure 15 - Timeline of the complete process of *rigor mortis* (Mean ± SEM) in Wistar Han rats. ($n=11$).

4.3.2. Post mortem body posture evaluation

Post mortem Wistar Han body posture was recorded photographically over time (Figure 16). The maximum rigidity was observed between 3 and 4 hours *post mortem*, in agreement with previously recorded RM data (Figure 14 and 15).

Time <i>post mortem</i> (h)							
0	0,5	1	2	3	4	18	24
							

Figure 16 – *Post mortem* body posture timeline in Wistar Han rats ($n=11$).

4.3.3. *Post mortem* ocular changes evaluation

Similar to mice, rats' ocular changes begin with a whitish cornea 30 minutes after CRA (36 ± 12 min, Figure 17B) and evolves to complete opacity (Figure 17C) during the fifth hour *post mortem* (241 ± 16 min). Mild sinking of the eyeball (Figure 17D) occurs earlier, two hours *post mortem* (123 ± 14 min), and evolves to total sinking twenty hours *post mortem* (1184 ± 13 min, Figure 17E).

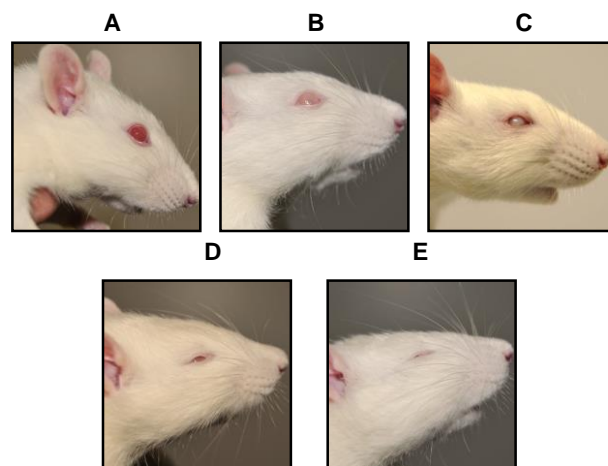


Figure 17 – *Post mortem* ocular changes in Wistar Han rats. A – Normal eye; B- Mild opacity; C – Total opacity; D – Mild sinking of the eyeball E- Total sinking of the eyeball ($n=11$).

4.3.4. *Algor mortis* evaluation

Body temperature measurements were assessed in controlled room temperature and relative humidity conditions ($RT = 21 \pm 0,1$ °C and $RH = 29 \pm 0,3$ %; $n=186$). The rats' body cooling rate was higher in the first hour after CRA, but their body temperature equalled room temperature 5 hours *post mortem* ($5 \pm 0,1$ h, Figure 18). The initial body temperature was $35,7 \pm 0,4$ °C but it decreased to $29,7 \pm 0,2$ °C in the first hour, reaching $22,2 \pm 0,3$ °C five hours *post mortem*. These results indicate that rats' body temperature decrease rate exhibits an exponential decay.

By nonlinear regression analysis, we were able to obtain the mathematical equation of the exponential cooling curve, shown in Figure 18.

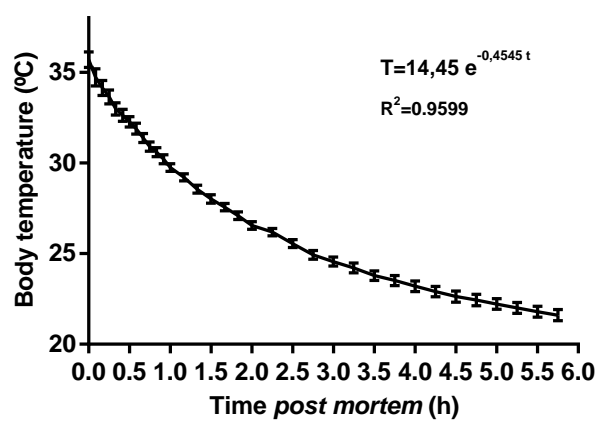


Figure 18 - *Post mortem* body temperature timeline in Wistar Han rats (*mean ± SEM, n=10*)
 T=body temperature (°C) and t=time(hours).

5. DISCUSSION / CONCLUSIONS

Our experimental findings indicate that in normal temperature and relative humidity room conditions for a rodent animal house facility *rigor* was fully developed 3,5 hours *post mortem* in CD1 mice ($3,3 \pm 0,2$ h, Figure 9A) and Wistar Han rats ($3,1 \pm 0,2$ h, Figure 14A) and 3 hours *post mortem* ($2,8 \pm 0,3$ h, Figure 4A) in C57BL/6 mice. The sequence of establishment of *rigor* was craniocaudal, similar to the downward direction observed in other species (DiMaio & DiMaio 2001, Munro & Munro 2008, Saukko & Knight 2016). The tail was an exception, since it developed full *rigor* before the hindlimbs in the three rodent strains used in this study. The resolution process was completed 22 hours *post mortem* ($21,5 \pm 0,2$ h, Figure 9B) in CD1 mice and 25 hours *post mortem* in Wistar Han rats ($24,1 \pm 0,3$ h, Figure 14B) and C57BL/6 mice ($24,9 \pm 0,2$ h, Figure 4B). This phase did not follow a craniocaudal direction, being the TMJ the last joint to lose its rigidity. So RM development in rodents does not seem to be affected by muscle volume, like Kobayash and colleagues demonstrated in 2000 (Kobayashi *et al.* 2001). These authors counteracted Shapiro's 1950's hypothesis that stated that (in humans) RM progresses downwards from the temporomandibular joint (TMJ) because it is a physico-chemical process that takes place simultaneously in all muscles, occurring first in small masses of muscle than in large masses. So a relatively small joint, such as the TMJ, surrounded by a moderate amount of muscle would become immobilised sooner than a large joint surrounded by a relatively large mass of muscle. This theory lacked experimental demonstration and has been overtaken by the current, that states that some of the probable factors that may explain RM sequence are: 1) the proportion of red fibres and white fibres in individual muscles; 2) the characteristic dynamic of each joint and 3) the differences in temperature that each muscle endures. So muscle volume would not affect the sequence of RM, with the exception of its interference with the muscle's temperature, to some extent (Kobayashi *et al.* 2001).

Our results seem to be in agreement with previous data published by Krompecher in 1981 and 1994 in rats, using a complex apparatus to measure the intensity of *rigor* at different ambient temperatures (6 to 24°C) (Krompecher 1981, Krompecher 1994). In those studies he concluded that the intensity of RM in rats increases in a room temperature dependent manner. At 6°C rat RM was established 48-60 hours *post mortem* and resolution was complete 168 hours after CRA; at 24°C those times decreased to 5 (RM establishment, RME) and 16 hours (RM resolution, RMR) and at 37°C RME occurred 3 and RMR 6 hours *post mortem* (Krompecher 1981). In 2013 Ozawa evaluated the rat soleum muscle stiffness as a reflection of RM intensity during time, in controlled temperature conditions. Stiffness was defined as the increase in tension (measured with a tension transducer system) in response to stretch and allowed the authors to demonstrate that muscle stiffness progression was quicker at higher temperatures. At 10°C the time required to reach 90%

of maximum stiffness was 9.26 ± 1.92 hours, at 25°C 3.85 ± 0.44 hours and at 37°C 1.79 ± 0.29 hours (Ozawa *et al.* 2013). In our experimental condition, using a manual method based in arbitrary units (a.u.), we concluded that with a room temperature of $21 \pm 0.1^{\circ}\text{C}$ ($n=186$) RM was established 3,5 hours *post mortem* ($3,1 \pm 0,2$ h, Figure 14A) and resolution was complete, approximately 24 hours after CRA ($24,1 \pm 0,3$ h, Figure 14B). These values are in the same timeline that Krompecher and Ozawa's described, accordingly to the relationship between room temperature an RM establishment / intensity and have the advantage that do not require special equipment (can be obtained manually) or training.

Another similarity between our and other studies is that RM was evaluated several times in a successive manner, which could lead to the breaking of *rigor* and its restauration with an inferior intensity (Krompecher 1994, Saukko & Knight 2016). We do not believe that this was the case, since flexing and extending the joints required little force and as soon as some resistance was noticed, the procedure was stopped. Furthermore, several authors that studied the process of *rigor mortis* in rats included in their protocols the administration of muscle relaxants (like mephenesin) prior to euthanasia (Krompecher 1981, Kobayashi *et al.* 2004). Their intention was to control some physical activity that precedes death, but this practice may involve both protocol (is does not mimic real animal conditions) and welfare (it is not humane to paralyse respiratory muscles before CRA) issues. So, in our study animals were gently manipulated previously to intraperitoneal pentobarbital administration and remained in their cages until CRA. After CRA all rodents were placed in a flat surface in dorsal recumbence to avoid the possibility of animal position interfere with the process of *rigor mortis*, a connexion that was already discarded by Kobayashi *et al.* (2004) (Kobayashi *et al.* 2004).

Regarding *post mortem* ocular changes, it should be noted that the position of the eyelids (open or closed) is crucial in the development of these changes. So when an animal dies in his sleep, it usually maintains the eyelids closed. Closed eyelids will have an impact on the progress of ocular changes, particularly in the case of corneal opacification since it delays the process of desiccation of the cornea (Calabuig *et al.* 2005, Saukko & Knight 2016). In this study, the animals maintained their eyelids open after death. Ocular changes were evaluated at defined time points and a score (0 to 4) was attributed considering de degree of corneal opacity and eye sinking. In both mice strains the process of opacification of the cornea was completed within the first hour *post mortem*, but the total sinking of the eyes occurred later, approximately 19 hours after CRA. In Wistar rats, total cornea opacification was delayed (to approximately 4 hours *post mortem*) but total eye sinking was only evident nearly 20 hours after CRA. These results are difficult to compare, since the majority of the literature discusses human data and do not take into consideration if the eyelids were open (Salam *et al.* 2012, Kawashima *et al.* 2015) or consider it an exclusion criterion (Kumar *et al.* 2012). Nevertheless, in those studies the authors considered

the transparency of cornea (from transparent to hazy and opaque) to estimate the TOD. They noticed that in the first 12 to 18 hours *post mortem* corneal turbidity increased, but it only reached maximum opacity more than 36 hours after death was declared (Kumar *et al.* 2012, Salam *et al.* 2012).

But besides studying RM and *post mortem* ocular changes in rodents we also studied *algor mortis*, the decrease in body temperature that occurs from death until the moment the corpse equals its temperature to ambient temperature. It has been a useful tool to estimate *post mortem* interval (PMI) in human forensics, but during the last 100 years several temperature - based methods tried to estimate the TOD, but none of them alone define the PMI with absolute precision (Henriquez *et al.* 2013). In this study we decided to evaluate rodents' *algor mortis* using a subcutaneous microchip, placed in the right flank. Our results indicate that initial body temperature (measured immediately after CRA) was lower than the reported physiological values for these species: 36,5 to 38 °C in mice and 35,9 to 37,5 °C in rats (Hrapkiewicz *et al.* 2013). In our experimental conditions, the initial body temperature of C57BL6 mice was $34,3 \pm 0,6$ °C (Figure 8), in CD1 mice $34,6 \pm 0,9$ °C (Figure 13) and in Wistar Han rats $35,7 \pm 1,3$ °C (Figure 18). This could be explained by the method of euthanasia, since after IP administration of a barbiturate overdose animals' activity decrease until they became immobile and CRA takes place (AVMA 2013), negatively influencing the initial body temperature measurements. Regarding the process of *post mortem* cooling, our data indicates that C57BL/6 mice took $3 \pm 0,4$ hours (Figure 8) and CD1 mice $3,1 \pm 0,2$ hours (Figure 13) until their body temperature equal room temperature ($21 \pm 0,1$ °C). In Wistar Han rats this process was slower, being completed $5 \pm 0,1$ hours *post mortem* (Figure 18), in same room temperature conditions. All groups showed an exponential decay of body temperature, so it was possible for us to obtain the mathematical equation that expresses each strain *post mortem* cooling curve (Figures 8, 13 and 18). This analysis allowed us to understand that the temperature gradient coefficient is higher in mice when compared to rats. These results could be explained by these species body weight differences, since smaller individuals will cool more rapidly than larger individuals in the same set of conditions (Goof, 2009). Since ambient conditions (temperature and relative humidity), cause of death and position of the animals (dorsal recumbence with direct contact with a flat surface in a coated chipboard table) were equivalent, it seems plausible to attribute this difference to rodents' body weight characteristics. Some forensic researchers mentioned the existence of a plateau in the *post mortem* cooling curve of human cadavers (Smart & Kaliszan 2012), but in this study we didn't observe that phenomena, in accordance with other *algor mortis* studies performed in animals (Jones 1982, Kaliszan *et al.* 2005, Erlandsson & Munro 2007, Henriquez *et al.* 2013). Moreover, our results regarding rats (305 ± 88 g) *post mortem* cooling curve are in agreement with previous published data, since in 1982 Peter Jones described that rats (230-270g) take approximately 6

hours *post mortem* until their body temperature matches room temperature. They used a subcutaneous platinum resistance thermometer, but the room temperature was unknown. Recently, another investigation group reached to a different conclusion, using a transesophageal probe; they demonstrate that rats (~400g) body temperature decreased *post mortem*, reaching room temperature (~20°C) 11 hours (ranging between 8 and 17 hours) after CRA (Henriquez, 2013). In our opinion this apparent discrepancy is not due to slight animal weight differences between the studies, but may be explained by the method used to measure body temperature; Jones and our investigation group used subcutaneous probes while Henriquez and colleagues used a deep transesophageal probe. We decided to evaluate rodents' body temperature by the implantation of subcutaneous microchip transponders because it was proven earlier that subcutaneous temperature is representative of rectal temperature (Cilia *et al.* 1998, Chen & White 2006), and one of the most common methods used worldwide to measure rats and mice body temperature.

During this study we realized that estimating the time of death of a deceased animal in the early *post mortem* period is a challenge and like others have noted before, it should not rely on only one of the previously mentioned *post mortem* alterations (Henssge & Madea 2004). Providentially, in rodent animal house facilities the animals live in a very controlled environment and the population characteristics (like body weight, for example) tends to be uniform within particular specie and strain. These are optimal conditions to perform this kind of studies, since the principal limitation to estimate the TOD in human cadavers is the multitude of conditions that these could be subject *post* and *ante mortem* and the variable characteristics of each individual (Henssge & Madea 2007). Considering that the basic conditions to accommodate rodents are internationally recognized (National Research Council 2011, Commission Recommendation 2007/526/EC of 18 June 2007), we decided to plot our experimental results to a summary timeline chart (Figure 19). We believe that with this chart it is easier to interpret our data concerning rodent RM and ocular *post mortem* changes over time, considering that these animals are daily monitored (so our timeline would not need to exceed 24 hours *post mortem*).

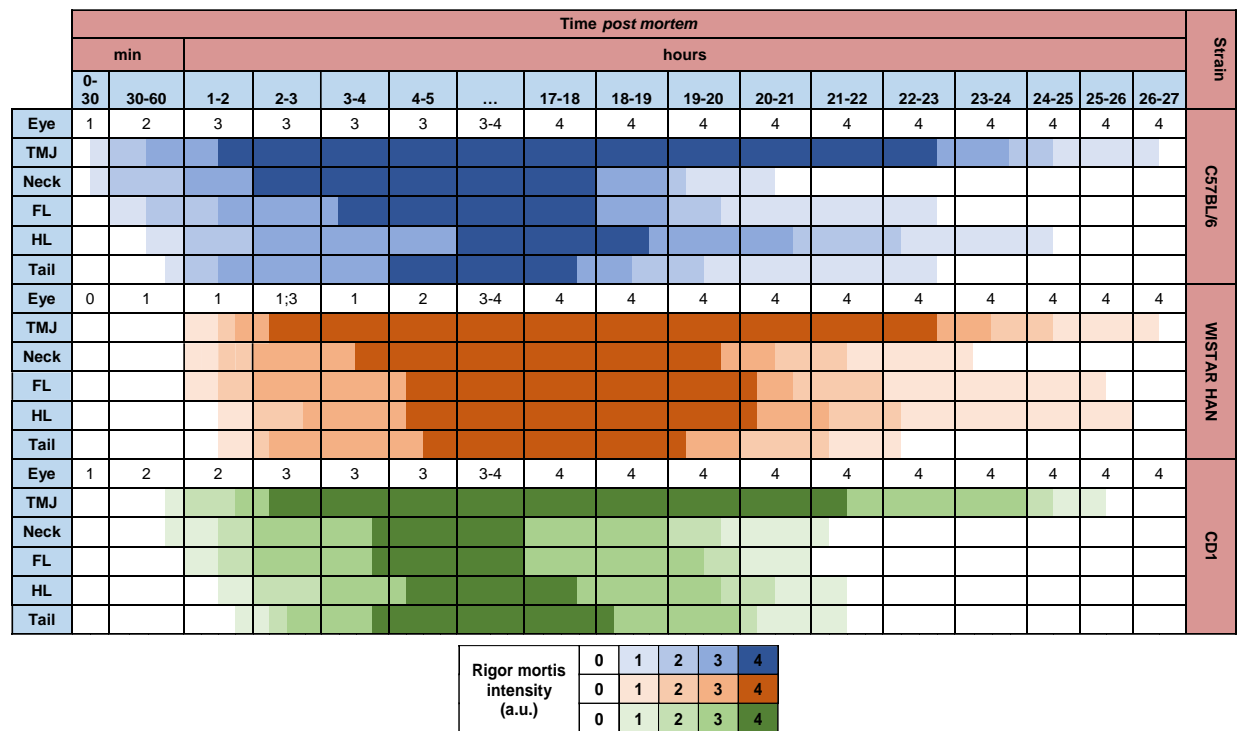


Figure 19 – *Rigor mortis* and ocular changes evaluation time table for three strains of rodents (C57BL/6 mice, CD1 mice and Wistar Han rats). (a.u. – arbitrary units)

Using the above chart, it is possible to estimate TOD using some data that everyone can collect when a rodent is found death in an animal facility: ocular changes and joint rigidity intensity. In order to maintain this chart accessible to all, we decided to exclude our *algor mortis* results, since rodents' body temperature measurement using a subcutaneous probe is not easily available.

We believe that this method can be used in a daily basis by animal technicians and researchers to determine rodents' TOD. This information may be very useful to some researchers to refine their protocol and hopefully it will contribute to increase the information that we can obtain from each animal, reducing the total number of mice and rat used in research.

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Attachments

How do you tell how long has a mouse been dead? *Rigor mortis* as a tool to estimate mice time of death (TOD) in animal house facilities

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In rodent animal house facilities knowing how to estimate the time of death (TOD) is of the utmost importance. *Rigor mortis* is defined as the stiffening of muscles after death and it has been used as a tool to estimate the TOD. [1] Interestingly, it is difficult to find correct information regarding *rigor mortis* onset, development and resolution in mice. Considering these difficulties, we decided to determine *rigor mortis* evolution in *Mus musculus* under stable environmental conditions.

Materials and methods

All animals were housed and maintained in accordance with Portuguese DL n° 113/2013. All animals were kept under stable temperature ($20 \pm 1^\circ\text{C}$, $n=228$) and relative humidity ($26,9 \pm 4,49\%$, $n=228$) conditions. Adult male and female C57BL/6 mice ($n=11$) with 4 ± 1 months of age and weighing 26 ± 2 gr were used in this study. Cardio-respiratory arrest (CRA) was induced by a single intraperitoneal administration of pentobarbital sodium (300 mg/Kg). Rigor was evaluated by flexing and extending the temporomandibular joint (TMJ), neck, front limbs (FL), hind limbs (HL) and tail and given a score from 0 to 4 according to the degree of rigidity. The eyeball was observed as well and given a score according to the degree of corneal opacity and sinking. Measurements were made during the first five hours and between the 17th and 27th hour *post mortem*.

Results

The results show that TMJ was completely blocked in first hour *post mortem* ($0,88 \pm 0,19$ h), followed by the neck ($1,33 \pm 0,41$ h) and front limbs ($1,90 \pm 0,74$ h) during the next hour. The tail ($2,24 \pm 0,75$ h) and hind limbs ($2,67 \pm 1,15$ h) were completely rigid before the end of the third hour *post mortem*. Muscle stiffness was maintained for approximately nineteen hours *post mortem*. The reversion process is initiated by the neck ($19,68 \pm 0,45$ h) followed by the tail ($20,30$

$\pm 1,38$ h), front limbs ($22,23 \pm 0,91$ h), hind limbs ($23,5 \pm 1,16$ h) and finally the TMJ at ($24,91 \pm 0,74$ h). The process is completed before the end of the 26th hour *post mortem*.

The cornea becomes totally opaque during the first hour ($48,64 \pm 15,97$ min) *post mortem* and this process begins as early as 15 minutes *post mortem* ($17,27 \pm 6,17$ min). Mild sinking of the eyeball is observed before the end of the second hour *post mortem* ($82,73 \pm 35,19$ min). This evolves to total sinking of the eyeball before the end of the 22nd hour *post mortem* ($1140 \pm 129,67$ min).

Discussion and conclusions

These results suggest that finding a mouse with *rigor mortis*, under typical animal house facility environmental conditions, indicates that the TOD occurred 4-24 hours ago. But, if animal house technicians perform a simple evaluation of TMJ, neck, FL, HL, tail movement and eyeball changes, is possible to understand if an animal died 2 hours ago (stiff TMJ, medium RM in other joints and opaque eye) or if the rigor is resolving (18-26 hours post mortem).

[1] Saukko P and Knight B (2016) "The pathophysiology of death" in (ed.) **Knight's Forensic Pathology**, 4th Edition, CRC Press, 55-93

[2] Mathur A and Agrawal Y K (2011) "An overview of methods used for estimation of time since death." **Australian Journal of Forensic Sciences** 43(4), 275-285

How do you tell how long has a mouse been dead? Rigor mortis as a tool to estimate mice time of death (TOD) in animal house facilities

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INTRODUCTION

Rigor mortis (RM) is defined as the stiffening of muscles after death. This occurs as a consequence of ATP depletion and the formation of rigid links between actin and myosin which are only broken by autolytic processes [1]. The onset and duration of *rigor mortis* are affected by numerous factors including temperature, physical exercise, age, muscular mass and existing ante-mortem pathologies. There are numerous reports of its use as a tool to estimate the time of death (TOD) [2]. In 1981, Krompecher obtained reference values for the establishment of *rigor mortis* in rats [3] but to our knowledge there's no mention in the literature regarding standardized values for mice. Since its widely use in biomedical research, it is of the utmost importance to obtain reliable methods to estimate the time of death of this species.

AIM

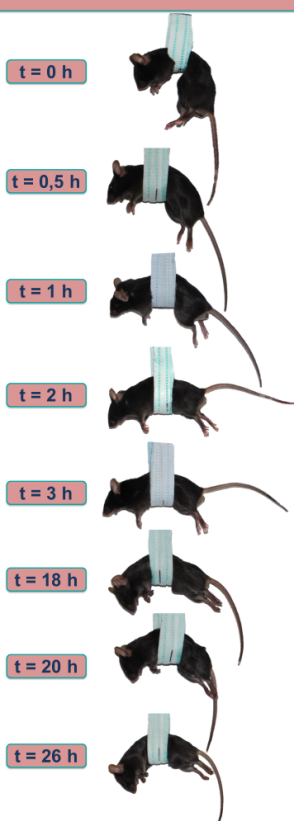
The aim of this study was to obtain reference values about *rigor mortis* evolution in *Mus musculus* under constant temperature/humidity conditions.

MATERIALS AND METHODS

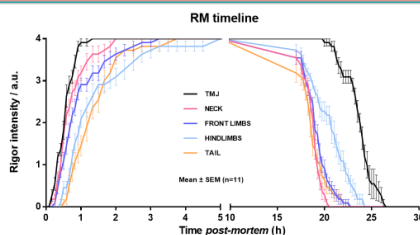
All animals were housed and maintained in accordance with Portuguese DL n° 113/2013. All animals were kept under stable temperature ($20,8 \pm 0,1^\circ\text{C}$, $n=257$) and relative humidity ($27,16 \pm 0,27\%$, $n=257$) conditions. Adult male and female C57BL/6 mice ($n=15$) with 4 ± 2 months of age and weighing 26 ± 3 gr were used in this study. Cardio-respiratory arrest (CRA) was induced by a single intraperitoneal administration of pentobarbital sodium (300 mg/Kg). Rigor was evaluated by flexing and extending the temporomandibular joint (TMJ), neck, front limbs (FL), hind limbs (HL) and tail and given a score from 0 to 4 according to the degree of rigidity. The eyeball was observed as well and given a score according to the degree of corneal opacity and sinking. Body temperature measurements were obtained by a subcutaneous microchip and the readings were taken with the Pocket Scanner System DAS 5007 (Bio Medic Data System [BMDS], Seaford, DE). Measurements were made during the first five hours and between the 17th and 27th hour after CRA.

RESULTS

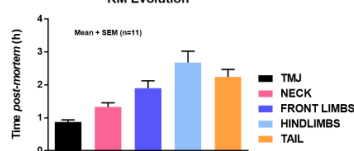
Post-mortem posture changes



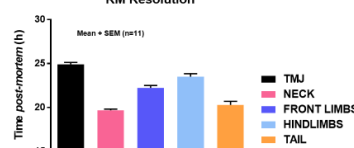
Rigor mortis (RM) evaluation



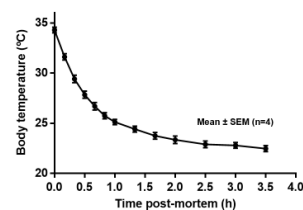
RM Evolution








RM Resolution



Post-mortem body temperature



Post-mortem ocular changes

Ocular changes (n=11)	Time post-mortem (min)	
0 (Normal)	0	
1 (Mild Opacity)	$17,27 \pm 1,85$	
2 (Total Opacity)	$48,64 \pm 4,81$	
3 (Mild Eye Sinking)	$82,73 \pm 10,60$	
4 (Total Eye Sinking)	$1140 \pm 39,10$	

	Time post-mortem (min)		Time post-mortem (h)																	
	0-30	30-60	1-2	2-3	3-4	4-5	...	17-18	18-19	19-20	20-21	21-22	22-23	23-24	24-25	25-26	26-27			
Eye	1	2	3	3	3	3	3-4	4	4	4	4	4	4	4	4	4	4			
TMJ																				
Neck																				
FL																				
HL																				
Tail																				
	Rigor intensity (a.u.)							0	1	2	3	4								

CONCLUSION

These results suggest that finding a mouse with *rigor mortis*, under typical animal house facility environmental conditions, indicates that the TOD occurred 4-24 hours ago. But, if animal house technicians perform a simple evaluation of TMJ, neck, FL, HL, tail movement, eyeball changes and body temperature, is possible to understand if an animal died 2 hours ago (stiff TMJ, medium RM in other joints and opaque eye) or if the rigor is resolving (18-26 h post mortem) by simply consulting the above summary chart.

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